Progression of Mycosis Fungoides Is Associated with Increasing Cutaneous Expression of Interleukin-10 mRNA

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Cytokines are believed to play an important role in the pathogenesis of cutaneous T cell lymphoma. Data regarding the local cytokine pattern in mycosis fungoides (MF) are partly conflicting. Recent studies have suggested a shift from type 1 to type 2 cytokine pattern because IL-4 and IL-5 mRNA have been more frequently detected in lesions of advanced stages. Another study has described a type 1 cytokine pattern in MF lesions. None of the previous studies of cytokine mRNA expression in MF, however, used quantitative methods, and therefore only the presence of a cytokine, but not the level of expression, could be determined. To gain better insight into the development of cytokine pattern during tumor progression we used semiquantitative reverse transcriptase-polymerase chain reaction to analyze cytokine mRNA expression in MF skin lesions at different stages. Biopsies from patients with patch (n = 11), plaque (n = 6), and tumor (n = 3) stage MF were compared with biopsies from patients with pleomorphic T cell lymphoma (n = 5), psoriasis (n = 7), atopic dermatitis (n = 5), and nonlesional skin (n = 8). MF progression was associated with significantly higher IL-10 and lower interferon-γ mRNA expression. Moreover, the stage-dependent increase in IL-10 mRNA expression was also found in paired samples from individual patients. Unlike in pleomorphic T cell lymphoma, however, typical T helper 2 cells did not seem to be the source of increasing IL-10 in advanced MF, because stage-independent IL-4 mRNA was rarely detected, suggesting contribution of nonlymphoid cells to local IL-10 production. The overexpression of IL-10 in MF may be of importance for tumor progression, because this immunosuppressive cytokine might be involved in downregulation of immunologic tumor surveillance. *Key words: cytokines/CTCL/IFN-γ/Th1/Th2. J Invest Dermatol 107:833–837, 1996*

In 1986 Mosmann and colleagues reported that $CD4^+$ T cells in mice can be classified into two subgroups with regard to their cytokine production pattern and the related functional activities, i.e., T helper 1 (Th1) and T helper 2 (Th2) cells (Mosmann *et al*, 1986). Th1 and Th2 cells are now defined by their predominant production of IL-2/interferon-γ (IFN-γ)/tumor necrosis factor-β or IL-4/IL-5/IL-6/IL-10/IL-13, respectively, and because not only $CD4^+$ Th cells contribute to the cytokine response, the cytokine patterns are often classified as type 1 or type 2. Although this model has provided a valuable framework for investigation of many immune reactions, recent studies have demonstrated a remarkable diversity in cytokine profiles, suggesting a continuous spectrum in which the classic type 1 and type 2 patterns represent extremes (reviewed by Kelso, 1995).

This diversity is also reflected in several dermatoses. For example, psoriasis, initially thought to be characterized by a typical type 1 pattern (Uyemura *et al*, 1993; Schlaak *et al*, 1994), exhibits a cytokine profile that is different from both types 1 and 2 (Vollmer *et al*, 1994). In atopic dermatitis, IL-10 (type 2) overexpression is accompanied by IFN-γ expression (type 1), whereas IL-4, a type 2 cytokine, is barely detectable (Ohmen *et al*, 1995). These findings illustrate the need for more extensive studies of cytokine expression using quantitative methods in order to characterize the cytokine patterns of dermatoses and better understand the pathophysiologic mechanisms.

Cytokines are considered to be of major importance for the pathogenesis of cutaneous T-cell lymphoma (CTCL) (Hsu *et al*, 1993; Rook *et al*, 1993; Burg *et al*, 1995). Several cytokines have been detected in mycosis fungoides (MF) skin lesions (Tron *et al*, 1988; Lawlor *et al*, 1990; Wismer *et al*, 1994; Sarris *et al*, 1995). Saed *et al* (1994) reported that plaque stage MF was characterized by a type 1 cytokine profile, whereas Vowels *et al* (1994), who investigated patch, plaque, and tumor stage MF, also detected IL-4 and IL-5 mRNA in some plaque stage and all tumor stage biopsies and therefore suggested a stage-dependent shift from the type 1 to type 2 in MF progression. This discrepancy may result, at least partially, from differences in sensitivity of the reverse transcriptase-polymerase chain reaction (RT-PCR) methods used (Lessin *et al*, 1995; Saed *et al*, 1995). Moreover, the use of qualitative, rather than quantitative, RT-PCR techniques has limited all cytokine gene expression studies in MF and other dermatoses.

IL-10 has been shown to have great impact on immunoregula-

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Abbreviations: AU, arbitrary unit; MF, mycosis fungoides; RT, reverse transcriptase; Th, T helper.

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tion. It favors the development of a type 2 cytokine pattern by inhibiting the IFN-γ production by T cells and natural killer cells via suppression of IL-12 synthesis in accessory cells (D'Andrea *et al*, 1993). Moreover, it suppresses proinflammatory cytokine production and reduces antigen-presenting capacity of monocytes/macrophages (De Waal Malefyt *et al*, 1991; Fiorentino *et al*, 1991a, b). Therefore, if enhanced IL-10 expression were present in the course of MF, it might contribute to the inhibition of an adequate anti-tumor response. So far, however, IL-10 transcripts have been sought in plaque lesions of MF but have not been detected (Saed *et al*, 1994).

The objective of this study was to further characterize the type 1/2 cytokine pattern in CTCL using a semiquantitative RT-PCR technique. We studied the expression of IL-10, IFN-γ, and IL-4 in patch, plaque, and tumor stage biopsies of MF patients including paired samples from single patients with skin lesions from different stages of the disorder. We demonstrate that the progression of MF is associated with increasing IL-10 and decreasing IFN-γ mRNA expression. Since, unlike IL-10 mRNA, IL-4 mRNA was only rarely detected, however, our results do not give sufficient evidence for the development of a classic type 2 cytokine pattern during MF progression.

MATERIALS AND METHODS

Patients Skin punch biopsies (4 to 8 mm) were obtained from adult patients with untreated CTCL (n = 11), psoriasis (n = 7), or atopic eczema (n = 5). The biopsy was divided and the diagnosis was confirmed by histology. In five of the 11 CTCL patients, diagnosis was further supported by the detection of monoclonal T-cell expansion using PCR technique. The material obtained from the CTCL patients consisted of samples from patch (n = 11), plaque (n = 6), tumor (n = 3) stage MF, and from pleomorphic T-cell lymphoma (n = 5) as determined by clinical and histologic criteria (Sterry, 1985). In eight patients (two MF, three psoriasis, three atopic dermatitis), an additional biopsy was obtained from apparently nonlesional (healthy) skin. The study was approved by our Institutional Review Board, and informed consent was obtained from all patients.

RNA Preparation and Reverse Transcription Total RNA was prepared from small tissue samples as described elsewhere (Platzer *et al*, 1994). Briefly, tissue specimens were snap-frozen in 500 μl of guanidinium isothiocyanate solution (Chomczynski and Sacchi, 1987) and homogenized with a dispersing system (Ultra Turrax, Janke and Kunkel, Staufen, Germany) during thawing. Five hundred microliters of water-saturated phenol, 100 μl of chloroform:isoamyl alcohol (24:1 vol/vol), and 50 μl of sodium acetate (2 M, pH 4) were added to the lysate. Mixtures were vortexed and stored on ice for a further 10 min prior to centrifugation (1000 × *g*, 20 min, 4°C). The aqueous phase was incubated with 8 μl of matrix (Rnaid PLUS Kit, Dianova, Hamburg, Germany) at room temperature for 5 min and then centrifuged (1000 × *g*, 1 min). The RNA-bonded matrix was washed three times with RNA wash solution (Rnaid PLUS Kit), and total RNA was eluted with 10–20 μl of diethyl pyrocarbonate-treated water. The quality of total RNA was estimated by electrophoresis on ethidium bromide–stained 1% agarose gel. mRNA was reverse transcribed into complementary DNA (cDNA) using oligo-dT primers (Murphy *et al*, 1993) in 40-μl reaction mixtures containing about 0.2 μg to 1 μg of total RNA.

RT-PCR Amplification and Primers Competitive PCR was introduced in 1989 (Wang *et al*, 1989) and was performed as we described in detail elsewhere (Platzer *et al*, 1994; Siegling *et al*, 1994). In our method a competitor control fragment is used together with sample cDNA in the reaction mixture. Sample and control cDNA are amplified with the same primers, and variations in amplification efficiency between reactions can be estimated because the control fragment serves as an internal control. The control and sample PCR products are distinguished on gel electrophoresis by differences in length. With the input concentration of the control fragment being known and amplification of both PCR products occurring proportionally, it is possible to quantify the sample cDNA. A cDNA equivalent of about 5 ng of total RNA was taken for each RT-PCR analysis. For some additional analyses a cDNA equivalent of up to 25 ng of total RNA was taken. The following oligonucleotides were used as primer pairs (sense/anti-sense): GADPH, GCAGGGGGGAGCCAAAAGGG/TGCCAGCCCCAGCGTCAAAG; IL-4, GCTTCCCCCTCTGTTCTTCC/TCTGGTTGGCTTCCTTCACA; IFN-γ, TCGTTTTGGGTTCTCTTGGC/GCAGGCAGGACAACCATTAC; IL-10, CTGAGAACCAAGACCCAGACATCAAGG/CAATAAGGTTTCTCAAGGGGCTGG (Platzer *et al*, 1994). In order to equilibrate the input cDNA amounts of the samples studied, we first determined the concentration of the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA using competitive RT-PCR and GAPDH-specific primers. After adjusting all samples to equal GAPDH–cDNA concentration, the relative cytokine cDNA amount was determined in a second competitive RT-PCR using the appropriate cytokine-specific primers. The relative concentration of the cytokine cDNA in each sample was expressed in arbitrary units (AU). One AU was defined as the lowest concentration of control fragment that yielded a detectable amplification product given the particular primer pair and PCR conditions used (Platzer *et al*, 1994). Control RT-PCR without template DNA was performed in all experiments to exclude contamination. As an additional control, the RT-PCR reaction was performed as described, but RT was omitted in the reaction mixture for each specimen in order to distinguish the amplification of contaminating genomic DNA from that of reversely transcribed mRNA. In order to increase sensitivity, RT-PCR without control fragments and high amounts of cDNA stock solutions (equivalent of 25 ng of total RNA) were performed in some experiments.

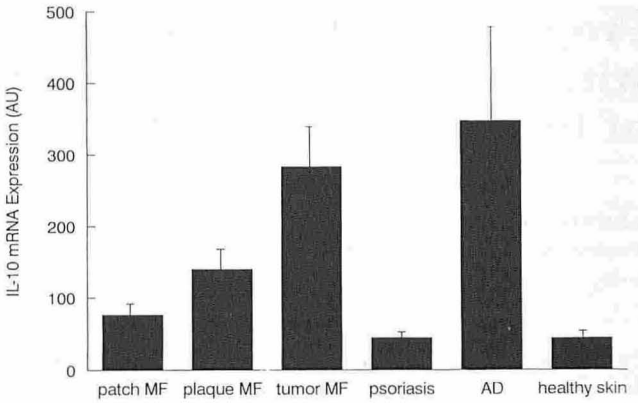

Figure 1. Progression of MF is associated with increasing IL-10 mRNA expression in skin lesions. IL-10 mRNA expression was analyzed in biopsies from patients with patch (n = 11), plaque (n = 6), or tumor (n = 3) stage MF and in biopsies from patients with psoriasis (n = 7) and atopic dermatitis (n = 5). Moreover, nonlesional (healthy) skin samples of eight patients (two MF, three psoriasis, three atopic dermatitis) were analyzed. RNA preparation, reverse transcription, and semiquantitative RT-PCR were carried out as described in *Materials and Methods* to determine IL-10 gene expression. The concentrations were expressed in AUs. One AU was defined as the lowest concentration of control fragment that yielded a detectable amplification product given the particular primer pair and PCR conditions used (Platzer *et al*, 1994). Data are shown as mean ± SEM. IL-10 mRNA was detected in almost all skin biopsies. The mean value of IL-10 mRNA expression in the more advanced stages of MF (plaque and tumor stage) was significantly higher than in patch stage MF ($p < 0.05$; Mann-Whitney test) and psoriatic or nonlesional skin ($p < 0.01$; Mann-Whitney test).

Statistical Analysis Data are presented as mean ± SEM. Statistical analysis included the chi-square assay for frequencies, the Mann-Whitney test for the comparison of groups, and the Wilcoxon test for paired samples. An error probability (p) lower than 5% was considered significant.

RESULTS

IL-10 mRNA Overexpression in MF Is Stage-Dependent IL-10 mRNA was detected in almost all skin samples. High levels of IL-10 mRNA were detected in skin from patients with atopic dermatitis and CTCL in contrast to healthy and psoriatic skin. Interestingly, the cutaneous IL-10 gene expression in MF was stage-dependent; levels of IL-10 mRNA expression increased along with disease progression from patch to plaque and from plaque to tumor stage **(Fig 1)**. When comparing the IL-10 transcription levels of patch stage MF (n = 11; mean ± SEM: 76 ± 22 AU) with that of more advanced stages (plaque and tumor; n = 9; 188 ± 39.6 AU), we found a significant difference ($p < 0.03$; Mann-Whitney test). IL-10 mRNA expression in advanced stages of MF as well as that in atopic dermatitis was significantly higher than in psoriatic

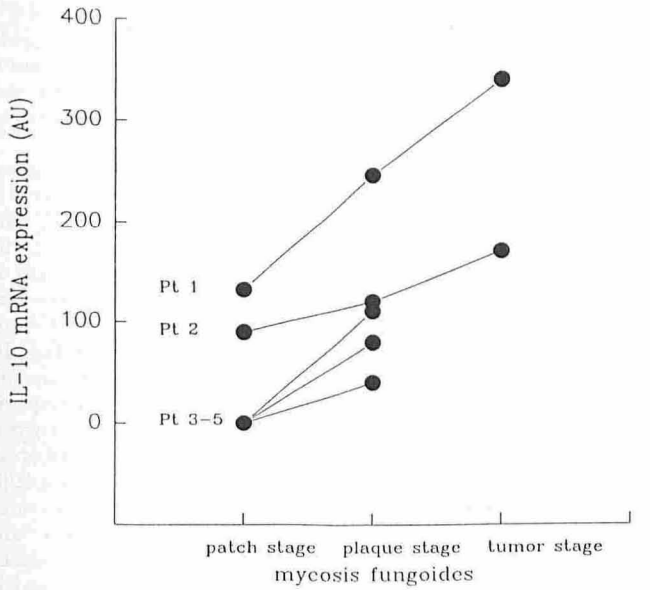

Figure 2. IL-10 gene expression in paired samples from MF patients. IL-10 mRNA expression was analyzed in paired skin biopsies of different stage lesions in five patients (Pt 1–5) with MF as described in **Fig 1**. The IL-10 mRNA concentration in patch stage was significantly lower than in plaque stage MF ($p < 0.05$; Wilcoxon test).

and nonlesional skin ($p < 0.01$). No significant differences were detected between patch stage MF, psoriatic, and nonlesional skin.

In five MF patients, paired biopsies from lesional skin of different stages were collected at the same time. In all five patients an increase in IL-10 mRNA expression from patch to plaque stage was demonstrated **(Fig 2)**; it was significantly higher in plaque stage lesions compared to patch lesions ($p < 0.05$; Wilcoxon test). Moreover, an further increase of IL-10 mRNA expression was detected in the patients in whom additional tumor stage biopsies had been analyzed **(Fig 2)**. **Figure 3** shows IL-10 mRNA expression in paired samples from a single patient. Also, in patients with pleomorphic T-cell lymphoma, IL-10 mRNA expression was

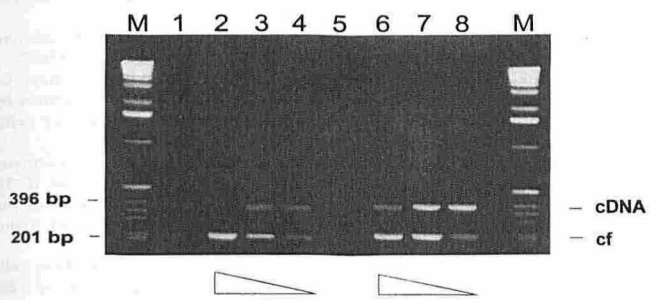

Figure 3. Higher IL-10 mRNA expression in tumor stage lesion than in patch stage lesion in MF. IL-10 mRNA expression was analyzed in biopsies from a single patient with patch and tumor stage MF lesions as described in **Fig 1**. The *triangles at the bottom* indicate the dilution of the control fragment DNA (cf): from *left to right*, 900 AU, 300 AU, 100 AU. The *upper and the lower bands* represent the cDNA and the control fragment amplification products, respectively. M, molecular weight markers. *Lane 1 and 5*, water blanks (negative control); *lanes 2–4*, patch stage sample with different cf dilutions; *lanes 6–8*, tumor stage sample with different control fragment dilutions. The equimolarity between control fragment and sample was reached in the patch and tumor stage sample at cf dilutions of about 100 AU and 300 AU, respectively. The video imaging analysis showed a 2.6-fold higher IL-10 level in the tumor stage sample than in the patch stage sample.

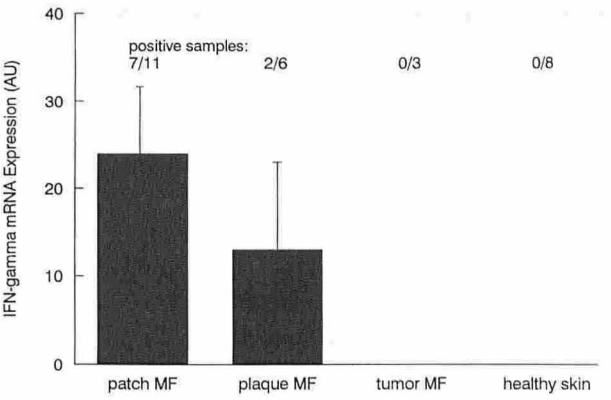

Figure 4. Progression of MF is associated with decreasing IFN-γ mRNA expression in skin lesions. IFN-γ mRNA expression was analyzed in biopsies from patients with patch, plaque, and tumor stage MF and nonlesional skin as described in **Fig 1**. Data are shown as mean ± SEM. The *numbers in the upper part* of the figure indicate the frequency of IFN-γ mRNA detection. The frequency of IFN-γ detection was significantly higher in patch stage MF than in more advanced stages (tumor and plaque; $p < 0.05$; chi-square assay) as well as in nonlesional skin ($p < 0.01$, chi-square assay).

higher in nodular lesions (n = 3; 810 ± 387 AU) than in patch lesions (n = 2; 220 ± 199 AU) (not shown).

MF Progression Is Associated with Decreasing IFN-γ mRNA Expression In contrast to IL-10, IFN-γ mRNA was detected in only a few MF samples and not in **uninvolved** skin. Interestingly, a decrease in IFN-γ mRNA expression from patch to plaque and from plaque to tumor stage was observed **(Fig 4)**. IFN-γ mRNA was detected more frequently in patch stage (seven of 11) than in the more advanced plaque and tumor stages (two of nine) of MF ($p < 0.05$; chi square assay) or nonlesional skin (zero of eight; $p < 0.01$, chi square assay). Moreover, the mean value of IFN-γ mRNA expression was about 2 times higher in patch MF than in plaque MF. The stage dependency in paired samples from each patient was not as strong as for IL-10 (not shown). In patients with pleomorphic T-cell lymphoma, IFN-γ mRNA expression was higher in patch lesions (n = 2; 500 ± 501 AU) than in nodular lesions (n = 3; 253 ± 129 AU) (not shown).

IL-4 mRNA Is Rarely Found in MF The stage-dependent inverse regulation of IL-10 and IFN-γ gene expression in MF skin suggested a shift from a type 1 to a type 2 cytokine pattern with progression of the disease. If the expansion and/or increased activation of Th2 cells was responsible for increasing IL-10 expression, IL-4 transcripts should be upregulated concomitantly.

Surprisingly, IL-4 mRNA was only rarely detected. Positive results were obtained in only one biopsy, which was from a patient with patch stage MF, but all samples of nodular pleomorphic T-cell lymphoma and three of five biopsies from patients with atopic dermatitis contained IL-4 mRNA **(Table I)**. The highest levels of IL-4 mRNA were detected in the nodular skin lesions from the patients with pleomorphic T-cell lymphoma. No significant differences between the groups were observed in the frequency of detection and the level of expression. The frequency of IL-4 detection in our samples did not increase when we used higher input cDNA amounts for the RT-PCR analysis (see *Materials and Methods*).

DISCUSSION

IL-10 mRNA was detected in all stages of MF. We found increasing levels of IL-10 mRNA expression, however, from patch to plaque and from plaque to tumor stage MF. Interestingly, this stage-dependent increase of IL-10 mRNA expression was also observed in paired samples from individual patients. These data

Table I. IL-4 Is Rarely Detectable in MF Skin Samples[a]

	Frequency of IL-4 Detection	IL-4 mRNA level (AU)	
		Median	Range
Mycosis fungoides			
Patch stage	1/11	0	0–28
Plaque stage	0/6	0	0
Tumor stage	0/3	0	0
Pleomorphic T cell lymphoma			
Patch-like	0/2	0	0
Nodular	3/3	63	45–82
Psoriasis	0/7	0	0
Atopic dermatitis	3/5	19	0–36
Nonlesional skin	0/8	0	0

[a] IL-4 mRNA expression was analyzed in skin biopsies. RNA preparation, reverse transcription, and semiquantitative RT-PCR were carried out as described in *Materials and Methods* to determine IL-4 gene expression. One arbitrary unit (AU) was defined as the lowest concentration of control fragment that yielded a detectable amplification product given the particular primer pair and PCR conditions used (Platzer *et al*, 1994).

support the advantage of semiquantitative methods. Conversely, IFN-γ gene expression decreased with progression of MF. Similar findings regarding IL-10 and IFN-γ expression were observed in pleomorphic T-cell lymphoma.

Thus far, these findings support the hypothesis that the progression of CTCL is associated with a switch from type 1 to type 2 cytokine pattern as suggested by others (Vowels *et al*, 1994; Lessin *et al*, 1995). While Saed *et al* (1994, 1995) initially presumed that MF exhibits a type 1 cytokine pattern, Vowels *et al* (1994) speculated that tumor cells in CTCL principally express the Th2 type and that the switch in the skin cytokine pattern results from an altered cell pattern during progression: in early stage MF, when only few tumor cells are detectable in the infiltrates, the cytokine profile is dominated by type 1 cytokine-expressing reactive T cells, whereas in advanced lesions type 2 malignant cells replace the reactive T-cell infiltrates. Unlike Vowels *et al*, however, we rarely detected IL-4 mRNA. This could be the result of a lower sensitivity of our IL-4 test system. The sensitivity of our quantitative RT-PCR method, however, was sufficiently high to detect about 10–20 IL-4 mRNA molecules per PCR tube (Platzer *et al*, 1994). In agreement with this relatively high sensitivity, we detected IL-4 mRNA in skin from nodular pleomorphic T-cell lymphoma and atopic dermatitis **(Table I)**. Moreover, IL-4 mRNA was easily detected in small kidney core biopsy specimens from transplant patients with acute rejection even though only 10–20% of graft cells belonged to the T-cell lineage (Ode-Hakim *et al*, 1996). This indicates that our test system is suitable for detection of IL-4 mRNA. Therefore, it was certainly sufficiently sensitive to exclude high level IL-4 gene expression in our samples from advanced stages of MF.

The apparent absence of significant IL-4 mRNA expression argues against typical Th2 cells as the source of the abundant IL-10 expression, which reached >1000 IL-10 cDNA molecules per 5 ng total RNA in advanced MF. Recently, a novel Th cell subtype was described that secretes TGF-β and IL-10 but not IL-4 and IL-5 or IFN-γ and IL-2. This Th subset is found particularly in the mucosa-associated lymphoid tissue and supports IgA synthesis (P. Matzinger, personal communication). We found a similar phenotype in kidney biopsies from patients with chronic rejection (Ode-Hakim *et al*, in press). The malignant cell in MF may also belong to this newly discovered Th phenotype. Expansion of these cells during tumor progression would explain increasing IL-10 expression in skin lesions. Interestingly, several other human tumors spontaneously secrete IL-10, thus downregulating the anti-tumor response (Pisa *et al*, 1992; Gastl *et al*, 1993; Lüscher *et al*, 1994). In addition to T cells (reactive or malignant), however, monocytes/macrophages and/or keratinocytes may also be responsible for increasing IL-10 gene expression in advanced MF, because these cells are also able to produce IL-10 after activation (Fiorentino *et al*, 1991a, b; Nickoloff and Turka, 1994; Enk *et al*, 1995). It is possible that as a result of the chronic inflammation in MF skin, IL-10 is overexpressed by keratinocytes. In this model, the malignant cells do not contribute, or contribute only marginally, to the local cytokine pattern. Further studies using *in situ* techniques are necessary to determine the cellular source of IL-10 and other cytokines detectable in MF skin.

We were able to confirm an association between tumor progression and a shift in the cytokine pattern, which might be involved in the pathogenesis of CTCL. Since IL-10 downregulates Th1 and cytotoxic T-cell function as well as dermal macrophage activity (Fiorentino *et al*, 1991a, b; Matsuda *et al*, 1994), this may result in the loss of control of the tumor by the immune system and progression of the tumor. An increasing number of advanced stage lesions in MF patients may result in systemic effects of locally produced IL-10, inducing global cytokine imbalance. The development of a systemic type 2–like cytokine pattern would explain the immunobiologic alterations occurring during CTCL progression. In the early stages, the immune response is usually unaltered. High level expression of IL-4 and IL-5 might explain eosinophilia and increased plasma levels of IgE, which are present in advanced stages of CTCL. Moreover, decreased natural killer cell and lymphokine-activated killer cell activity, as well as a decreased T-cell response to antigens and mitogens, have been described in advanced CTCL (Edelson, 1980). Interestingly, Dummer *et al* (1993) have shown that peripheral blood mononuclear cells in patients with nonleukemic CTCL showed a preferential secretion of type 2 cytokines upon stimulation (elevated secretion of IL-4 and decreased secretion of IFN-γ). In Sézary patients, similar results were found (Vowels *et al*, 1992), and IL-12 production was also found to be reduced (Rook *et al*, 1995). The question whether the overexpression of IL-10 induces this disturbance of immune regulation is of major importance, because this could support further tumor progression, including extracutaneous manifestations, and may contribute to the increased risk of development of second malignancy described in CTCL patients (Kantor *et al*, 1989).

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